Phosphoinositol 3-kinase-γ mediates antineutrophil cytoplasmic autoantibody-induced glomerulonephritis

Adrian Schreiber[1], Susanne Rolle[1], Ludmilla Peripelittchenko[2], Joerg Rademann[2,3], Wolfgang Schneider[1], Friedrich C. Luft[1] and Ralph Kettritz[1]

[1]*Department of Nephrology and Hypertension, Medical Faculty of the Charité, Franz Volhard Clinic, Max Delbrück Center for Molecular Medicine, HELIOS-Klinikum, Berlin, Germany;* [2]*Forschungsinstitut for Molecular Pharmacology, Berlin, Germany and* [3]*Institute of Chemistry and Biochemistry, Free University Berlin, Berlin, Germany*

Antineutrophil cytoplasmic autoantibodies (ANCA) are associated with necrotizing crescentic glomerulonephritis (NCGN) and systemic vasculitis. We examined the role of phosphoinositol 3 kinase-γ isoform (PI3Kγ) in ANCA-activated neutrophil functions. Further, we tested whether its inhibition protects a mouse model of ANCA NCGN from developing NCGN. We transplanted bone marrow from wild-type mice or PI3Kγ-deficient mice into myeloperoxidase-deficient mice immunized with myeloperoxidase. Bone marrow from PI3Kγ$^{-/-}$ mice protected against development of the disease. Similarly, bone marrow transplanted from wild-type mice followed by treatment with the specific PI3Kγ inhibitor AS605240 also protected these mice against NCGN in this model. AS605240 significantly abrogated myeloperoxidase- or proteinase 3-ANCA-stimulated superoxide production *in vitro*. Furthermore, ANCA-induced degranulation and GM-CSF-stimulated migration in a transwell assay of isolated human neutrophils were also abrogated by the drug. We found that PI3Kγ plays a pivotal role in ANCA-induced NCGN and suggest that its specific inhibition may provide a novel treatment target.

Kidney International (2010) **77,** 118–128; doi:10.1038/ki.2009.420; published online 11 November 2009

KEYWORDS: ANCA; animal model; crescentic glomerulonephritis; inflammation; neutrophils; PI3Kγ

Correspondence: *Adrian Schreiber, Department of Nephrology and Hypertension, Medical Faculty of the Charité, Franz Volhard Clinic, Max Delbrück Center for Molecular Medicine, Schwanebecker Chaussee 50, Berlin 13125, Germany. E-mail: adrian.schreiber@gmx.de*

Received 4 May 2009; revised 26 August 2009; accepted 15 September 2009; published online 11 November 2009

Patients with necrotizing crescentic glomerulonephritis (NCGN) and systemic small-vessel vasculitis feature antineutrophil cytoplasmic autoantibodies (ANCAs).[1,2] ANCAs activate neutrophils and are pathogenic in a mouse model.[3–9] ANCAs bind to ANCA antigens on the cell membrane of cytokine-primed neutrophils.[10–12] Signaling pathways are activated that mediate several functions.[3,6,11,13–20] The phosphoinositol 3-kinase (PI3K)/Akt pathway is pivotal for ANCA-induced respiratory burst activity.[13,15,21] The PI3K family is divided into four classes, namely, I_A, I_B, II, and III.[22] Class I is responsible for cell-surface-receptor-generated phosphatidylinositol 3,4,5-bisphosphate that regulates the nicotinamide adenine dinucleotide phosphate oxidase.[23] Class I PI3K consists of a regulatory and one of three different p110 catalytic subunits. Two class I subclasses are distinguished, namely, I_A and I_B. I_A enzymes have three types of p110 catalytic subunits: p110α and p110β, which are expressed in many tissues, whereas p110δ is expressed primarily in leukocytes. The single I_B enzyme, p110γ, is expressed preferentially in leukocytes. I_A enzymes are activated by protein tyrosine kinase receptors, whereas I_B enzyme is activated through G-protein-coupled receptors. PI3Kγ controls neutrophil respiratory burst, migration, and Akt activation in response to various ligands and *in vitro* experiments by Ben-Smith *et al.*[13,24–30] implicated the γ-isoform also in ANCA-mediated neutrophil activation. All of these functions participate in ANCA-mediated organ damage. Recently, the small-molecule PI3Kγ inhibitor AS605240 with more than 30-fold selectivity over PI3Kδ and PI3Kβ and 7.5-fold selectivity over PI3Kα was generated. AS605240 (1 μmol/l) blocked PI3Kγ-dependent Akt phosphorylation in primary monocytes, whereas PI3Kγ-independent Akt phosphorylation was not affected.[28] Furthermore, specific PI3Kγ inhibition blocked glomerulonephritis and improved survival in a lupus erythematosus mouse model.[31] AS605240 is orally active and 50 mg/kg twice a day suppressed rheumatoid arthritis in another mouse model.[28] We tested whether the PI3Kγ isoform controls neutrophil functions that mediate ANCA-induced NCGN. We found that PI3Kγ could be a treatment target for ANCA-induced NCGN.

RESULTS

Bone marrow from PI3Kγ-deficient animals provides protection from ANCA-induced NCGN

We first tested the hypothesis that PI3Kγ has an important function in an ANCA-glomerulonephritis mouse model. Anti-myeloperoxidase (MPO)-mediated NCGN was induced by the previously described bone marrow (BM) transplantation approach.[32,33] MPO-deficient animals were immunized with MPO, irradiated, and transplanted with BM cells from either wild-type (WT) mice or PI3Kγ gene-deficient ($PI3K\gamma^{-/-}$) animals ($n=8$ in each group). At 8 weeks after transplantation, mice were killed. All mice (100%) transplanted with WT BM developed hematuria and proteinuria, whereas mice transplanted with $PI3K\gamma^{-/-}$ BM did not develop significant urine abnormalities. These results were confirmed by a higher urinary albumin excretion in the mice transplanted with WT BM compared with the $PI3K\gamma^{-/-}$ BM group (1047.0 ± 783.5 μg/ml albumin in the WT mice versus 203.9 ± 67.4 μg/ml in the $PI3K\gamma^{-/-}$ mice). The values did not reach statistical significance because of high interindividual variability.

All mice transplanted with WT BM developed NCGN on histology (100% disease induction) whereas in the $PI3K\gamma^{-/-}$ BM group mice developed only weak glomerular abnormalities (six of the eight mice got weak glomerular disease, two did not develop any glomerular abnormalities). When these findings were quantitatively assessed, mice in the WT BM group showed on average $22.3 \pm 7.5\%$ crescents and $9.7 \pm 2.8\%$ necrosis whereas mice in the $PI3K\gamma^{-/-}$ BM group developed $3.1 \pm 1.1\%$ crescents and $2.5 \pm 0.3\%$ necrosis ($P<0.05$ for both) (Figure 1). Immunohistology showed weak IgG, IgA, IgM, and C3 deposition that did not differ between both groups (data not shown). By enzyme-linked immunosorbent assay we found similar anti-MPO titer in both experimental groups (WT group: d0 1.25 ± 0.38 arbitrary units (AUs), d56 0.69 ± 0.44 AU; $PI3K\gamma^{-/-}$ group: d0 1.22 ± 0.26 AU, d56 0.77 ± 0.22 AU) excluding the possibility that differences in anti-MPO titers were responsible for the protection from NCGN in the $PI3K\gamma^{-/-}$ mice. Furthermore, mice transplanted with WT BM showed slightly higher circulating white blood counts and neutrophil counts at d56, probably reflecting disease activity similar to what is seen in patients with active ANCA vasculitis (Table 1). Finally, both groups showed similar engraftment rates with MPO-positive circulating neutrophils (data not shown). These data establish that neutrophil PI3Kγ is important for NCGN induction by anti-MPO antibodies without affecting BM engraftment, anti-MPO titers, or the deposition of immunoglobulins and complement.

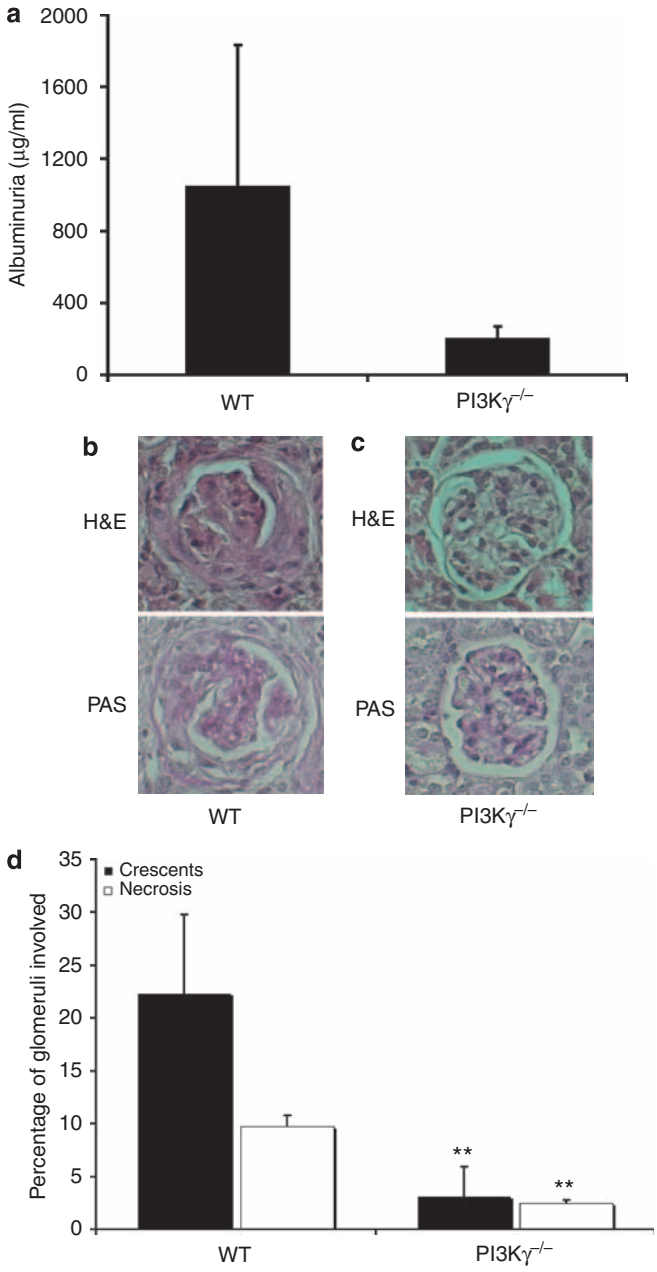

Figure 1 | Myeloperoxidase (MPO)-deficient animals were immunized with MPO, irradiated, and transplanted with bone marrow (BM) cells from either wild-type (WT) or phosphoinositide 3 kinase-γ isoform ($PI3K\gamma^{-/-}$) mice. Mice were killed 8 weeks after BM transplantation and albuminuria (**a**), as well as renal tissue was examined. WT BM-transplanted mice (**b**) developed glomerular crescents and necrosis, whereas $PI3K\gamma^{-/-}$ BM-transplanted mice (**c**) were protected. The extent of glomerular crescents and necrosis were expressed as the mean percentage of glomeruli with crescents and necrosis (**d**).

Glomerular neutrophil and macrophage influx was diminished in $PI3K\gamma^{-/-}$ Mice

To test whether PI3Kγ has an important function in leukocyte migration *in vivo*, we assessed glomerular neutrophil and monocyte/macrophage accumulation. The data show a reduced neutrophil and monocyte/macrophage influx in glomeruli from mice transplanted with $PI3K\gamma^{-/-}$ BM (Table 2). These findings indicate that PI3Kγ is needed for circulating leukocytes to accumulate in the glomeruli and induce NCGN.

Pharmacological PI3Kγ inhibition blocks ANCA-induced Akt phosphorylation in primed neutrophils

We showed earlier that ANCA stimulation results in a second Akt phosphorylation wave in cytokine-primed neutrophils.[15] We used two priming cytokines that differ in their ability to induce PI3Kγ. Tumor necrosis factor-α (TNFα) acts through G-protein-coupled-receptor-mediated PI3Kγ activation and granulocyte macrophage colony-stimulating factor (GM-CSF) activates RTK that does not induce PI3Kγ.[34–36] Accordingly, we observed significant inhibition of TNFα-induced Akt phosphorylation with 10 μmol/l LY294002 as a nonspecific PI3K inhibitor and with 0.5 μmol/l AS605240 as a PI3Kγ-specific inhibitor (Figure 2a). In contrast, LY294002 prevented Akt phosphorylation in GM-CSF-treated neutrophils whereas AS605240 did not. Western blot experiments indicate that 0.5 μmol/l AS605240 does not block p38 and extracellular signal-regulated kinase (ERK) phosphorylation underscoring the specificity for PI3Kγ inhibition (Figure 2b and c). Stimulation with human ANCA after cytokine priming caused a second strong Akt phosphorylation wave that was not observed with control IgG. Pretreatment with either LY294002 or AS605240 blocked Akt phosphorylation in both GM-CSF- and TNFα-primed neutrophils subsequently activated by human ANCA IgG (Figure 3). These findings show that ANCA-activated PI3K and that PI3Kγ isoform inhibition blocked Akt phosphorylation.

Table 1 | Peripheral blood cell analysis at the day of killing in MPO-deficient mice transplanted with WT or PI3Kγ$^{-/-}$ BM (Exp 1) and in VC-treated mice versus AS-treated mice (Exp 2) (data are means ± s.e.m.)

	WBC (Gpt/l)	HGB (mg/dl)	Lym (Gpt/l)	Mo (Gpt/l)	Gra (Gpt/l)
Exp 1					
WT	17.2 ± 10.0	13.4 ± 1.1	9.7 ± 5.2	1.9 ± 1.9	5.6 ± 3.9
PI3Kγ$^{-/-}$	11.7 ± 2.7	14.4 ± 1.0	7.0 ± 2.3	1.0 ± 0.4	3.5 ± 1.2
Exp 2					
VC	5.9 ± 1.2	12.3 ± 0.5	2.7 ± 0.7	0.3 ± 0.1	2.4 ± 0.6
AS	5.1 ± 0.3	13.6 ± 0.4	2.5 ± 6.0	0.5 ± 0.1	2.1 ± 0.3

Abbreviations: AS, AS605240; BM, bone marrow; MPO, Gpt/l, giga-particles/liter; Gra, granulocyte; HGB, hemoglobin; Lym, lymphocyte; Mo, monocyte; myeloperoxidase; PI3K, phosphoinositol 3-kinase; VC, vehicle control; WBC, white blood cell count; WT, wild type.
None of the comparisons showed significant differences.

PI3Kγ inhibition blocks ANCA-induced reactive oxygen species generation in neutrophils

We next tested whether PI3Kγ inhibition prevents respiratory burst by ANCA. We first titrated a dose-response study where cells were preincubated over an AS605240 range from 2×10^{-6} to 1×10^{-9} mol/l for 30 min followed by GM-CSF priming and treatment with a monoclonal antibody (mAb) to MPO (Figure 4a). On the basis of these data, neutrophils were then pretreated with 0.5 μmol/l AS605240, primed with GM-CSF or TNFα, and subsequently activated with human ANCA IgG. Incubation of TNFα- or GM-CSF-primed neutrophils with control IgG did not result in generation of superoxide, whereas stimulation with MPO-ANCA IgG or proteinase 3 (PR3)-ANCA IgG induced a strong respiratory burst (Figure 4b). Pretreatment with LY294002 or with AS605240 abrogated the ANCA-stimulated superoxide production in either TNFα- or GM-CSF-primed neutrophils ($n = 4$, $P < 0.05$). The difference between both inhibitors was not statistically significant. These data indicate that the PI3Kγ isoform is necessary for superoxide production by ANCA.

To investigate whether the reduction in superoxide generation was merely a consequence of reduced ANCA antigen expression, we performed flow cytometry experiments. mPR3 expression of resting neutrophils was 47.9 ± 4.9 mean fluorescence intensity that was upregulated with 2 ng/ml TNFα to 90.6 ± 12.8 mean fluorescence intensity. Treatment with 0.5 μmol/l AS605240 did not significantly change the membrane expression. We observed 79.6 ± 14.6 mean fluorescence intensity for TNFα-treated neutrophils ($n = 5$, n.s.). Expectantly, treatment with 10 μmol/l SB202190 significantly abrogated TNFα-induced membrane PR3 expression as showed in parallel experiments (90.6 ± 12.8 mean fluorescence intensity to 59.0 ± 11.2; $P < 0.05$; $n = 5$).

PI3Kγ inhibition blocks ANCA-induced neutrophil degranulation

We next tested whether PI3Kγ inhibition abrogates neutrophil degranulation after stimulation with ANCA. Neutrophils

Table 2 | Quantitated immunohistochemical microscopy for glomerular neutrophil and macrophage infiltration in MPO-deficient mice transplanted with WT or PI3Kγ$^{-/-}$ BM (Exp 1) and in VC-treated mice versus AS-treated mice (Exp 2)

	Neutrophils			Macrophages		
Group	% Positive Glom	Cells/+Glom	Cells/Glom	% Positive Glom	Cells/+Glom	Cells/Glom
Exp 1						
WT	36.9 ± 6.6*	1.4 ± 0.1	0.5 ± 0.1*	48.5 ± 8.0*	1.4 ± 0.1	0.7 ± 0.1
PI3Kγ$^{-/-}$	14.5 ± 4.0	1.4 ± 0.2	0.2 ± 0.1	22.5 ± 5.3	1.4 ± 0.1	0.3 ± 0.1
Exp 2						
VC	35.2 ± 11.0*	1.5 ± 0.1	0.5 ± 0.2	35.3 ± 12.4	2.4 ± 0.5	1.0 ± 0.5
AS	7.2 ± 2.0	1.3 ± 0.2	0.1 ± 0.0	12.1 ± 2.4	1.3 ± 0.2	0.2 ± 0.1

Abbreviations: AS, AS605240; BM, bone marrow; MPO, myeloperoxidase; PI3K, phosphoinositol 3-kinase; VC, vehicle control; WT, wild type.
Numbers represent mean numbers ± s.e.m. of positive glomeruli (% Positive Glom), mean numbers ± s.e.m. of positive cells per positive glomeruli (Cells/+Glom), mean numbers ± s.e.m. of positive cells per glomerular cross section (Cells/+Glom).
*$P < 0.05$.

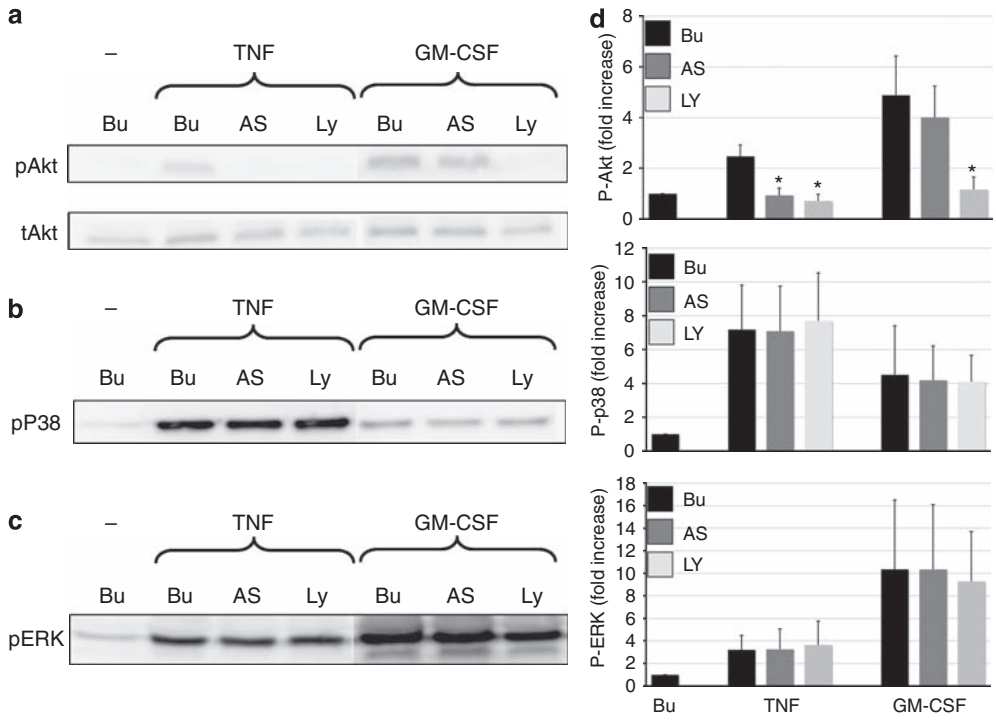

Figure 2 | Western blot analysis for phospho-Akt, phospho-p38 mitogen-activated protein kinase, and phospho-ERK is shown. Neutrophils were preincubated with 10 μmol/l LY294002 (Ly), 0.5 μmol/l AS605240 (AS), or dimethyl sulfoxide as a control (Bu). Cells were subsequently primed with buffer (–), tumor necrosis factor α (TNF), or granulocyte macrophage colony-stimulating factor (GM-CSF). After 10 min, samples were harvested and phosphorylated Akt (pAkt, **a**), phosphorylated p38 mitogen-activated protein kinase (pP38, **b**), and phosphorylated ERK (pERK, **c**) were determined by immunoblotting. A representative example of five independent experiments is shown. Total Akt (tAkt) is shown as loading control. The corresponding densitometric analysis is given in (**d**).

were pretreated with LY294002 or AS605240, followed by priming with GM-CSF or TNFα, and subsequently activated with human ANCA IgG. Degranulation of primary granules was assayed by β-glucuronidase release. As shown in Figure 4c, degranulation of TNFα- and GM-CSF-primed neutrophils stimulated with ANCA IgG was significantly inhibited by pretreatment with both PI3K inhibitors ($n=4$, $P<0.05$). These data show that activation of PI3Kγ isoform by ANCA IgG is essential for ANCA-induced neutrophil degranulation.

PI3Kγ inhibition blocks neutrophil migration

Glomerular neutrophil recruitment is a hallmark of ANCA-induced glomerulonephritis. When we tested whether specific inhibition of PI3Kγ blocks migration toward GM-CSF, we observed that neutrophil migration increased within the first 2 h reaching a plateau thereafter. Furthermore, a strong reduction of migration occurred with both LY294002 and AS605240 pretreatment (Figure 5). These data show that PI3Kγ controls neutrophil migration toward GM-CSF *in vitro*. In a second set of experiments, the effect of LY294002 and AS605240 on migration in the presence of human ANCA IgG was assessed. Compared with control IgG, GM-CSF-mediated migration was slightly decreased by MPO and PR3 ANCA ($n=6$, n.s.). However, LY294002 and AS605240 showed a similar inhibitory effect on migration in the presence of ANCA. For clarity, migration data after 2 h are shown in Figure 5b.

PI3Kγ inhibition does not influence spreading and adhesion

Other important neutrophil functions involved in the multistep process of glomerular neutrophil influx in ANCA-induced NCGN are adhesion to and spreading on extracellular matrices. After 120 min, we observed an increase in neutrophil adhesion to fibronectin with GM-CSF treatment from 1.6 ± 0.1 to $2.4\pm0.5\times10^4$ cells ($P<0.05$). The GM-CSF effect was not inhibited by LY294002 ($2.1\pm0.5\times10^4$) or AS605240 ($2.3\pm0.7\times10^4$, $n=5$, n.s.). We then assessed whether mAb to PR3 or MPO further increased GM-CSF-mediated adhesion. GM-CSF treatment in the presence of isotype control for 120 min resulted in $2.3\pm0.3\times10^4$, with anti-MPO mAb in $2.6\pm1.2\times10^4$ and with anti-PR3 mAb in $2.4\pm0.6\times10^4$ adherent cells. This increase was small and not statistically significant ($n=5$).

Furthermore, we found that GM-CSF treatment increased neutrophil spreading on fibronectin (from 8 ± 3 to $45\%\pm12$, $n=5$, $P<0.05$). The GM-CSF effect was not affected by LY294002 ($37\%\pm14$) or AS605240 ($39\%\pm14$, $n=5$, n.s.) pretreatment. GM-CSF treatment in the presence of isotype control resulted in $43\%\pm12$ with a variable effect by anti-MPO mAb ($57\%\pm10$) and anti-PR3 mAb ($72\%\pm8$) that was not statistically significant ($n=5$, n.s.).

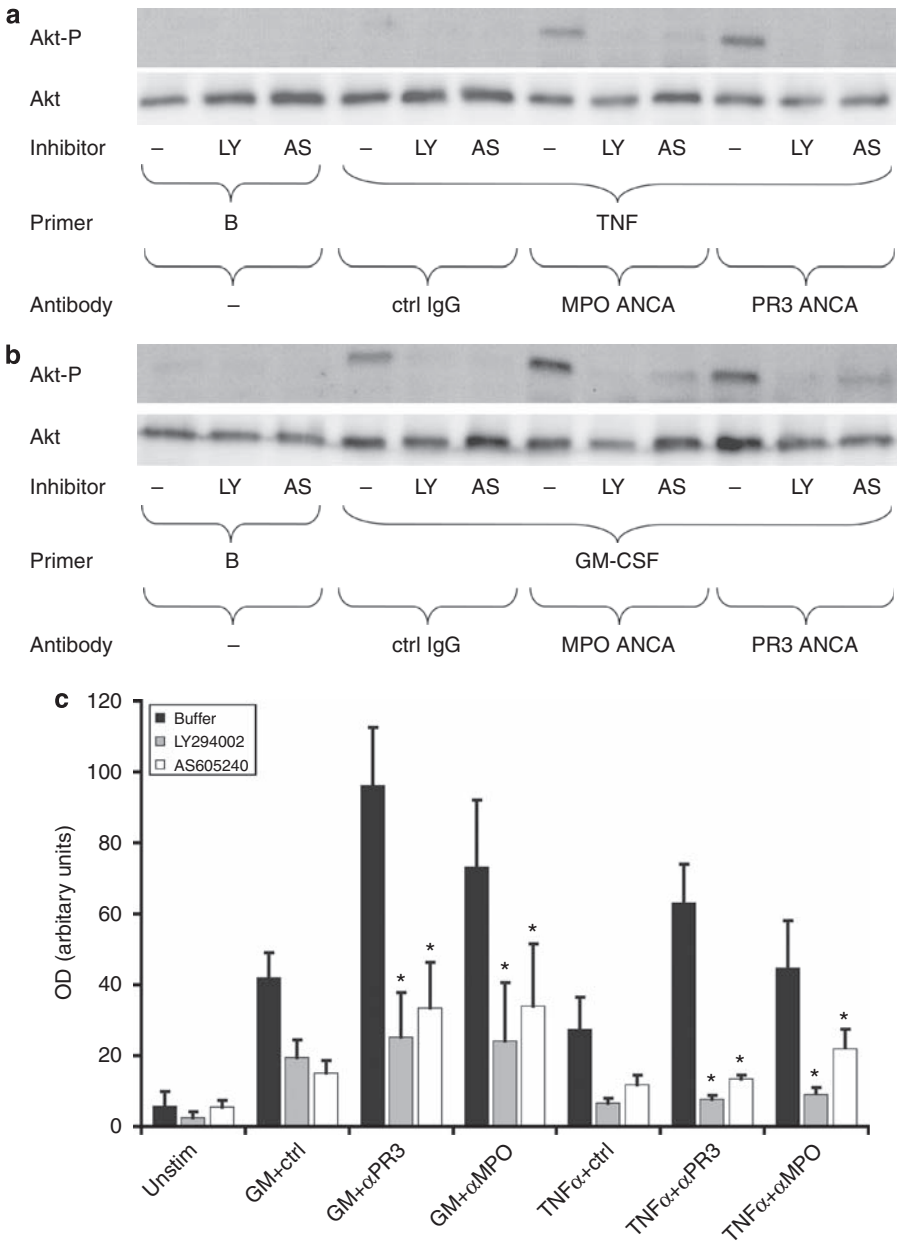

Figure 3 | Western blot analysis for phospho-Akt is shown. Neutrophils were preincubated with 10 μmol/l LY294002 (LY), 0.5 μmol/l AS605240 (AS), or dimethyl sulfoxide as a control (−). Cells were subsequently primed with buffer (B) or tumor necrosis factor α (TNF) (**a**) or buffer or granulocyte macrophage colony-stimulating factor (GM-CSF) (**b**) for 45 min, followed by stimulation with control IgG (ctrl), myeloperoxidase-antineutrophil cytoplasmic autoantibodies (MPO-ANCA), or PR3-ANCA. After 10 min, samples were harvested, S473 phosphorylated Akt was determined by immunoblotting, and a representative example of four independent experiments is shown in panels **a** and **b**. Total Akt is shown as loading control. The corresponding densitometric analysis is given in panel **c** ($n = 4$). These data show that LY294002 and AS605240 block ANCA-induced phosphoinositol 3-kinase (PI3K)/Akt activation.

Oral AS605250 treatment protects animals from ANCA-induced NCGN

We finally tested whether specific PI3Kγ inhibition by orally administered AS605250 provides a novel treatment option for ANCA-induced NCGN. Immunized MPO-deficient mice were irradiated and subsequently transplanted with WT BM cells. At day 21 after transplantation treatment with oral gavage twice daily containing 30 mg/kg body weight AS605250 or vehicle control for the next 5 weeks was started. We chose day 21 as a starting point to avoid interference with BM engraftment. At this time point, we already found engraftment with MPO-positive neutrophils in the circulation and the beginning of urine abnormalities.

At 8 weeks after BM transplantation, all mice transplanted with WT BM and treated with vehicle control showed hematuria and proteinuria, whereas AS605240-treated mice did not develop significant urine abnormalities. These results were confirmed by a higher urinary albumin excretion in the

mice treated with vehicle control in comparison to the AS605240-treated group: 118.2 ± 67.1 μg/ml in the control mice compared with 32.8 ± 15.1 μg/ml in the AS605240-treated group. The difference did not reach statistically significance.

All vehicle-control-treated mice developed NCGN on histology (100% disease induction) whereas AS605240-treated mice developed only weak glomerular abnormalities (three of the six mice developed weak glomerular disease, three did not develop any glomerular abnormalities). Mice in the control group showed on average 15.6 ± 3.9% crescents and 6.0 ± 1.5% necrosis, whereas mice in the AS605240 group developed 1.4 ± 0.6% crescents and 0.5 ± 0.4% necrosis ($P<0.01$ for both; Figure 6). Both groups showed similar anti-MPO titers by enzyme-linked immunosorbent assay (vehicle-control group: d0 1.25 ± 0.41 AU, d56 1.10 ± 0.64 AU; AS group: d0 1.39 ± 0.52 AU, d56 0.99 ± 0.83 AU), similar engraftment with MPO-positive cells (data not shown), and no difference in blood cell counts. These data establish that AS605240 did not exert toxic effects that interfered with BM engraftment (Table 1).

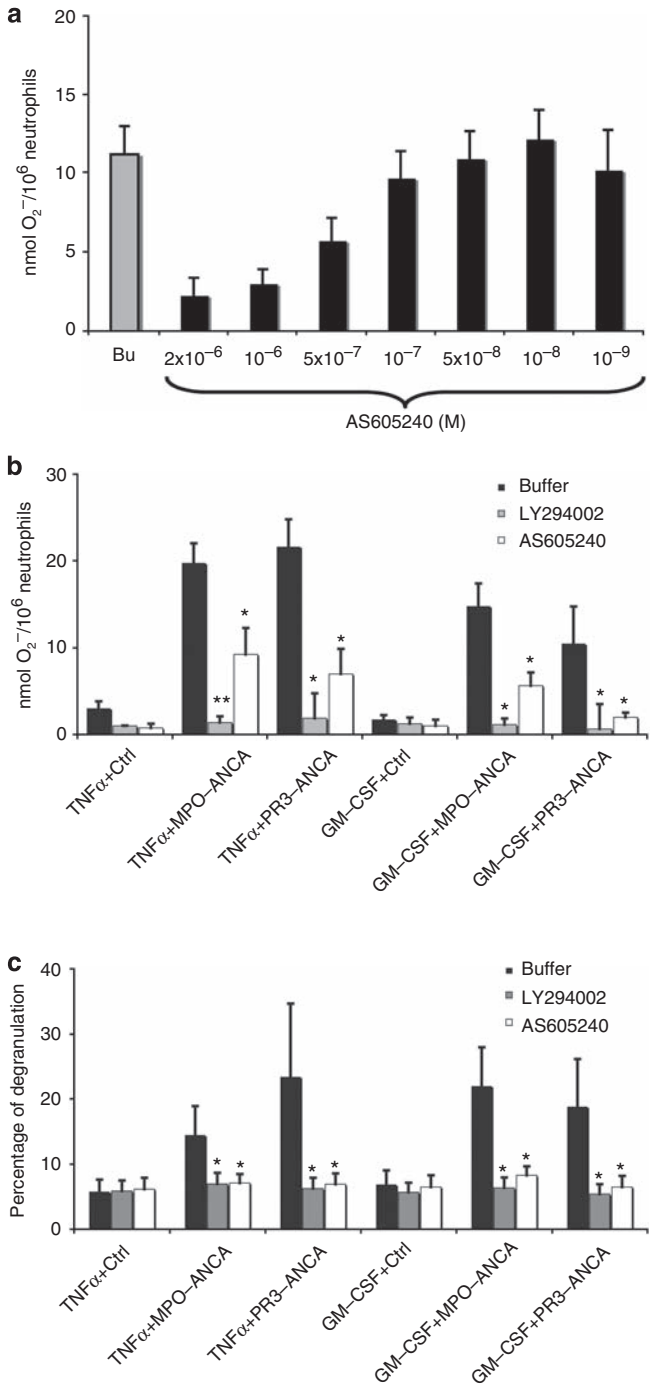

Figure 4 | We studied the inhibitory effect of LY294002 and AS605240 on antineutrophil cytoplasmic autoantibody (ANCA)-induced superoxide release and degranulation. Neutrophils were incubated with increasing concentrations of AS605240 for 30 min followed by 15 min priming with granulocyte macrophage colony-stimulating factor (GM-CSF) and subsequent treatment with a monoclonal antibody (mAb) to myeloperoxidase (MPO; $n=4$, **a**). After preincubation with 10 μmol/l LY294002, 0.5 μmol/l AS605240, or dimethyl sulfoxide control, cells were primed with either tumor necrosis factor α (TNFα) or GM-CSF followed by activation with control IgG (Ctrl), MPO-ANCA, or PR3-ANCA. Statistical analysis for the superoxide data is provided in (**b**) and for degranulation in (**c**) ($n=4$, $^*P<0.05$; $^{**}P<0.01$). In each of the four independent experiments, preparations from two patients with PR3-ANCA and two patients with MPO-ANCA were tested. These data show that LY294002 and AS605240 block ANCA-induced respiratory burst and degranulation.

Glomerular neutrophil and macrophage influx was reduced by AS605240 treatment

The glomerular neutrophil and macrophage accumulation in anti-MPO NCGN was diminished in mice treated with AS605240 as shown in Table 2, indicating that the blockade of PI3Kγ reduces *in vivo* glomerular neutrophil and monocyte/macrophage accumulation.

DISCUSSION

We used *in vivo* models to show that PI3Kγ controls neutrophil functions that participate in ANCA-induced glomerular damage. Our data suggest PI3Kγ inhibition is a new therapeutic approach in ANCA vasculitis. ANCAs induce NCGN that resembles the human disease in several animal models.[8,33,37] Glomerular neutrophil influx is a hallmark of ANCA-induced NCGN and neutrophils are the main effector cells.[38] Several neutrophil functions that are thought to participate in the process of glomerular injury are induced by ANCA, including superoxide generation,[3,7,39] degranulation,[3,19] and cell adhesion.[9,40] Elucidation of intracellular pathways controlling these effector functions will identify novel treatment targets. The more detailed the functional significance of these signal events is understood the more specific treatment can be designed.

We evaluated the importance of PI3Kγ in an *in vivo* ANCA model. We selected two different approaches, namely, primarily PI3Kγ gene-deleted mice and thereafter, oral

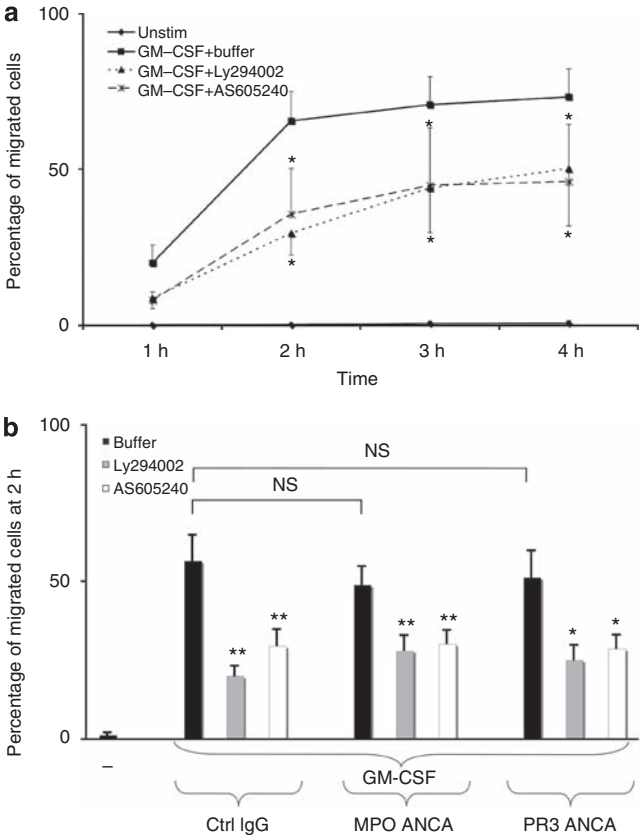

Figure 5 | The effect of LY294002 and AS605240 on neutrophil migration is shown. Neutrophils were preincubated with dimethyl sulfoxide (DMSO) control (Buffer), 10 μmol/l LY294002, or 0.5 μmol/l AS605240 and loaded in the upper well of a transwell device. The percentage of neutrophils migrated toward granulocyte macrophage colony-stimulating factor (GM-CSF) was quantified by the myeloperoxidase (MPO) assay over a time period of 4 h ($n = 5$, $^*P < 0.05$, **a**). In (**b**) neutrophils were preincubated with DMSO control, 10 μmol/l LY294002, or 0.5 μmol/l AS605240 for 30 min. Control IgG, MPO-antineutrophil cytoplasmic autoantibodies (ANCA), or PR3-ANCA were added and samples were loaded into the upper well of the chamber. The percentage of migrated cells responding to GM-CSF at 2 h is depicted ($n = 6$, $^*P < 0.05$, $^{**}P < 0.01$). These data show that LY294002 and AS605240 block cytokine-induced neutrophil migration in the absence and presence of ANCA.

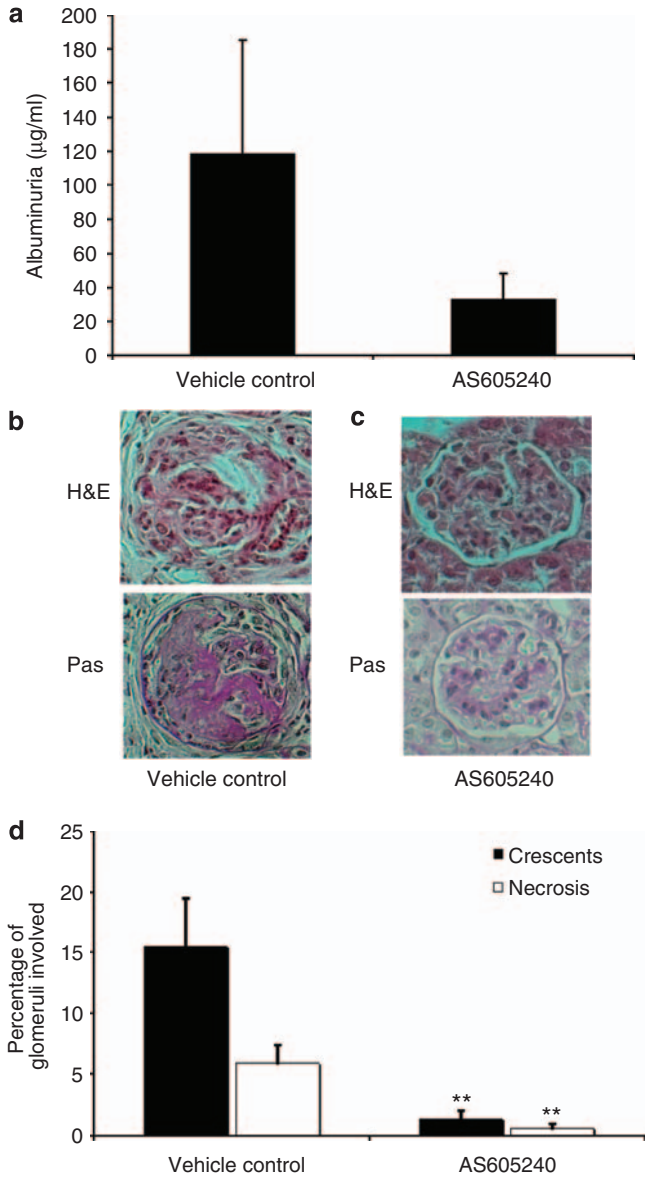

Figure 6 | Myeloperoxidase (MPO)-deficient animals were immunized with MPO, irradiated, and transplanted with wild-type (WT) bone marrow (BM) cells. Mice were orally treated with AS605240 or vehicle control from day 21 after transplant for 5 weeks. Mice were killed 8 weeks after BM transplantation and albuminuria (**a**) as well as renal tissue was examined. WT BM-transplanted mice treated with vehicle control (**b**) developed glomerular crescents and necrosis, whereas AS605240-treated mice (**c**) were protected. The extent of glomerular crescents and extent of necrosis were expressed as the mean percentage of glomeruli with crescents or necrosis (**d**; $^{**}P < 0.01$.).

treatment with a specific PI3Kγ inhibitor. The first approach was undertaken to provide a proof-of-principle study to firmly establish a function for the PI3Kγ isoform in ANCA-induced NCGN. We had an ideal option with our optimized mouse model, because the model involves BM transplantation from WT mice into irradiated MPO-immunized MPO-deficient mice.[32,33] Transplantation of BM from PI3Kγ-deficient mice instead of WT BM almost completely prevented NCGN in this neutrophil-dependent mouse model of ANCA-induced disease. These experiments, together with our *in vitro* data, strongly point toward an important function for PI3Kγ in neutrophils during ANCA-mediated NCGN induction. Because transplantation of PI3Kγ$^{-/-}$ BM deleted this isoform in all leukocytes, we cannot exclude an additional function for PI3Kγ in lymphocytes and monocytes. However, we observed no reduction in BM engraftment, anti-MPO titers, or circulating leukocyte numbers.

Our *in vitro* results show that ANCA-mediated activation of respiratory burst and degranulation, as well cytokine-initiated migration depends on the PI3Kγ pathway. Two classes of PI3K have been described in human neutrophils,

the phosphotyrosine-associated class I_A p85/p110 isoform and the G-protein-activated class I_B p101/p110γ isoform. We used two different cytokine primers in our *in vitro* experiments, TNFα as PI3Kγ-dependent primer and GM-CSF as PI3Kγ-independent primer, and showed that ANCA IgG itself activate PI3Kγ. In these studies, we used the PI3Kγ inhibitor AS605240, as described previously.[28,31] We substantiate and extend previous findings that ANCA activation of neutrophils involves the PI3K pathway.[13,15,41] Ben-Smith *et al.*[13] showed convincingly that ANCAs activate the PI3Kγ isoform. To our knowledge, our study is the first to show that specific inhibition of the PI3Kγ isoform blocks ANCA-stimulated responses *in vitro*. We selected 0.5 μmol/l AS605240, as based on our *in vitro* dose-response and specificity studies, and observed an approximately 50% inhibition of superoxide generation and in almost complete inhibition of degranulation. Conceivably, PI3Kγ participates more in the degranulation response compared with respiratory burst. In addition, both in the absence and presence of ANCA, we observed a strong decrease in neutrophil migration toward a chemotactic gradient of GM-CSF when PI3Kγ was blocked. These data are supported by published studies in PI3Kγ$^{-/-}$ mice whose neutrophils had impaired chemotactic response toward IL-8, fMLP, C5a, and MIP-1α *in vitro* and attenuated recruitment to the peritoneal cavity.[24–26,42]

We recently showed involvement of C5a and the neutrophil C5a receptor in ANCA-induced NCGN in mice.[32] We found that ANCA-induced neutrophil degranulation results in C5a generation and that C5a through the neutrophil C5a receptor accelerates ANCA-induced neutrophil activation. C5a is a strong chemoattractant and it was shown by others that C5a-induced signaling and functions in neutrophils, including migration, involves PI3Kγ.[43] Possibly, the reduction in glomerular neutrophil and macrophage influx that we observed in the PI3Kγ-deficient group is, at least in part, also a consequence of blocking C5a receptor signaling through PI3Kγ pathway. Our animal experiments establish the significance of PI3Kγ. However, transplantation of PI3Kγ-deficient BM is currently not a feasible option in patients.

We pursued a second *in vivo* approach to modulate PI3Kγ by applying a highly specific PI3Kγ-isoform inhibitor, namely AS605240. AS605240 is a small molecule that was successfully used intravenously to prevent disease in a mouse model of lupus nephritis and orally in a mouse model of arthritis.[28,31] On the basis of these results, we selected the oral administration route twice daily over 5 weeks, thereby mimicking the human situation. Oral treatment with AS605240 prevented ANCA-induced NCGN in our animal model. The drug was started 21 days after BM transplantation and did not interfere with engraftment and anti-MPO antibody production. At this early time point, the disease was approximately 5 weeks away from its culmination. We could envision such a situation at the beginning of a relapse. Whether starting treatment at later time points, when the disease is more active, has the same efficiency needs to be tested in future studies.

The perspectives for the patients are the pivotal function of PI3Kγ in ANCA-mediated neutrophil functions and thereby documented a potential drug target. We used PI3Kγ gene-deleted mice to prove that this PI3Kγ isoform controls ANCA-mediated NCGN. Finally, we showed that pharmacological PI3Kγ blockade abrogates the disease in an animal model. We believe that these findings may provide a novel treatment option in patients with ANCA vasculitis.

MATERIALS AND METHODS

Materials

TNFα and GM-CSF were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany). Phorbol-2-myristate-13-acetate and Ficoll-Hypaque were from Sigma-Aldrich (Deisenhofen, Germany). HBSS, phosphate-buffered saline, and Trypan blue were from Biochrom (Berlin, Germany) and dextran was purchased from GE Healthcare (Amsterdam, the Netherlands). LY294002 and SB202190 were from Calbiochem (Schwalbach, Germany). The PR3 mAb 4A5 was from Wieslab (Lund, Sweden), the anti-PR3 mAb (CLB12.8) from Hiss (Freiburg, Germany) and the mAb to MPO was from Acris (Hiddenhausen, Germany), phospho-specific antibodies to p38 mitogen-activated protein kinase, ERK, and Akt (S473) were from Cell Signaling (Frankfurt, Germany). The horseradish peroxidase-labeled donkey anti-rabbit IgG was from GE Healthcare. Endotoxin-free reagents and plastic disposables were used in all experiments.

Human neutrophils and human IgG

Neutrophils from healthy donors were isolated from heparinized whole blood as described previously.[11] Cell viability by Trypan blue exclusion was found to be >99% in every experiment. Wright-Giemsa staining revealed a neutrophil percentage >95%. Normal IgG and ANCA-IgG were prepared from normal volunteers and patients with active MPO and PR3-ANCA disease using a High-Trap-protein-G column in an Äkta-FPLC system (GE Healthcare).

Measurement of respiratory burst

Superoxide was measured using superoxide dismutase-inhibitable reduction of ferricytochrome c.[44] Cells (0.75×10^6) were preincubated with 5 μg/ml cytochalasin B for 10 min and primed with 2 ng/ml TNFα or 20 ng/ml GM-CSF for 15 min before human ANCA (125 μg/ml) were added. In some experiments, cells were pretreated for 30 min on ice with 10 μmol/l LY294002, the indicated AS605240 concentrations and dimethyl sulfoxide (DMSO) control, respectively. Samples were incubated in 96-well plates at 37°C for up to 60 min, and the absorption of samples with and without 300 U/ml superoxide dismutase was scanned repetitively at 550 nm using a Microplate Autoreader (Molecular Devices, Munich, Germany).

Flow cytometry to assess PR3 surface expression

Flow cytometry was used to evaluate the PR3 membrane expression. Cells were preincubated with the inhibitors or DMSO control for 30 min on ice, followed by treatment with 2 ng/ml TNFα or HBSS control for 15 min at 37°C. Thereafter, cells were washed and stained with an mAb to PR3 (CLB12.8) or an isotype control followed by a secondary fluorescein isothiocyanate-conjugated F(ab)2-fragment of goat anti-mouse IgG. Flow cytometry was performed using an FACScan, and 10,000 events per sample were collected.

Neutrophil adhesion and spreading

A total of 96-well plates coated with (10 μg/cm^2) fibronectin were used. Neutrophils (1×10^5) in 100 μl HBSS^{++} were either left untreated or were treated with 20 ng/ml GM-CSF. In some experiments, cells were pretreated for 30 min on ice with 10 μmol/l LY294002, the indicated AS605240 concentrations and DMSO control, respectively. When indicated, cells were treated with 10 μg/ml isotype control or mAb to PR3 and MPO, respectively. Adherent cells were estimated using the MPO assay. Briefly, adherent cells were lysed in 100 μl of 0.5% Triton-X-100. Substrate (100 μl; 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid; Sigma-Aldrich) was added and OD was read at 450 nm. OD of the experimental sample was compared with a standard curve that showed an excellent correlation between OD and cell number ($R^2 = 0.96$). For estimation of cell spreading, nonadherent cells were discarded and the percentage of spread cells was assessed. At least 100 cells of each sample were counted by two investigators using phase-contrast microscopy. Cells that were phase dark, enlarged, and irregular were considered to be spread.

Degranulation assay

Neutrophils (5×10^5) were preincubated with 5 μg/ml cytochalasin B and primed in a 96-well microtiter plate for 15 min with 2 ng/ml TNFα or 20 ng/ml GM-CSF. Stimulation was performed with 125 μg/ml PR3-ANCA or MPO-ANCA or human control IgG for up to 120 min. At 30-min intervals the cell-free supernatants were collected and β-glucuronidase activity was assessed by the cleavage of phenolphthalein glucuronic acid (Sigma-Aldrich). Each tube contained 50 μl of a 0.01 mol/l solution phenolphthalein glucuronic acid in 0.1 mol/l acetic buffer (pH 4.6) with 0.04% Triton X-100, and was mixed with 50 μl of the cell-free supernatant. OD values were measured at 405 nm. Nonstimulated neutrophils served as baseline, whereas the total β-glucuronidase content was obtained by incubation 5×10^5 neutrophils with 1% Triton X-100.

Transmigration assay

Migration was tested in fibronectin-coated transwells (3.0 μmol/l, 6.5 mm from Corning, NY, USA). Neutrophils (1.5×10^6) in HBSS^{++} were stimulated at 37°C with 5 ng/ml GM-CSF to transmigrate to the lower well. When indicated, cells were treated with 125 μg/ml PR3-ANCA, MPO-ANCA, and control IgG, respectively. For time-course studies, the upper chamber of the transwell device was gently moved to a new well in 60-min intervals. Transmigrated cells were quantified by MPO assay as described previously.[7]

Western blot analysis of phosphorylated Akt, p38 mitogen-activated protein kinase, and ERK

Samples were incubated for 5 min at 95°C in loading buffer (250 mmol/l Tris-HCl (pH 6.8) with 4% sodium dodecyl sulfate, 20% glycerol, 0.01% bromophenol blue, 10% β-mercaptoethanol). Protein (20 μg) was loaded, electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was blocked and incubated overnight with the indicated primary antibodies, followed by a horseradish peroxidase-labeled secondary antibody. Densitometry was performed with the Chemicapt 12.8 software using a Chemismart 5000 scanner (Vilber Lourmat, Eberhardzell, Germany).

Preparation of (*E*)-5-((quinoxalin-6-yl)methylene) thiazolidine-2,4-dione (AS605240)

Quinoxaline-6-carbaldehyde (1.04 g, 6.55 mmol, 1 equiv.; Invitrogen, Karlsruhe, Germany) and thiazolidine-2,4-dione (1.15 g, 9.82 mmol, 1.5 equiv.) were dried under vacuum with gentle heating in a 100 ml two-neck round bottom flask. The flask was equipped with a reflux condenser and all solids were dissolved in dry toluene (40 ml). Piperidine (0.6 equiv.) and acetic acid (0.6 equiv.) were added and the mixture was refluxed at an oil bath temperature of 115–120°C for 16 h. After cooling the formed precipitate was filtered off, washed with acetonitrile, water, and methanol and dried. The product was recrystallized from methanol to yield pale-yellow powder (m.p. 299°C (dec.), 0.95 g 57% yield): ^{1}H-NMR (DMF-d$_7$, 300 MHz) δ = 7.98–8.08 (m, 2H), 8.18–8.25 (m, 2H), 8.28–8.35 (m, 1H), 9.44 (s, 1H); ^{13}C-NMR (DMF-d$_7$, 75 MHz) δ = 124.52, 129.34, 129.59, 131.55, 131.59, 132.90, 141.63, 141.86, 148.25, 148.75, 167.61, 171.76. ESI-MS: *m/z* 258.1 (M + H)$^+$.

Mice

C57BL/6J (B6) mice breeding pairs were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice lacking MPO (MPO$^{-/-}$ mice) were the sixth-generation progeny of a backcross into B6 mice originally generated by Aratani *et al.*[45] MPO$^{-/-}$ mice (8- to 10-week old) were used for immunization. B6 WT mice and PI3Kγ$^{-/-}$ mice (9- to 10-week old; generous gift from Professor J Penninger, Research Institute of Molecular Pathology, Vienna, Austria) were used as BM donors. Local authorities approved all animal experiments, which followed American Physiologic Society guidelines for animal care.

Immunization of mice

The purification of mouse MPO and the immunization of MPO$^{-/-}$ mice were performed as previously described.[33] MPO$^{-/-}$ mice were immunized intraperitoneally with 10 μg of MPO in complete Freund's adjuvant. Antibodies were monitored by anti-MPO enzyme-linked immunosorbent assay. The presence of circulating anti-MPO antibodies was confirmed in selected animals by indirect immunofluorescence microscopy assay on murine neutrophils as described.[33]

BM transplantation in mice

After immunization the MPO$^{-/-}$ mice were kept at sterile housing conditions with food and water *ad libitum* (sterile water with trimethoprim and sulfadoxine, Borgal). One week after the last immunization, mice were gamma-irradiated with 900 rad of whole-body dose and reconstituted with BM from WT or PI3Kγ$^{-/-}$ mice of the same genetic background. BM cells were harvested from femurs and tibia, erythrocytes were lysed, and 1.5×10^7 BM cells were intravenously injected. Engraftment was measured by histochemistry for MPO activity on peripheral blood smears. In the treatment experiments at day 22 after BM transplantation, AS605240 or vehicle control (0.5% carboxymethylcellulose/0.25% Tween-20) was administered orally by gavage twice daily at a dose of 30 mg/kg body weight until killing at week 8. At killing, blood cell counts were carried out by a hematology analyzer (Scil Vet abc, Viernheim, Germany).

Functional evaluation of renal injury

Mice were placed in metabolic cages one day before killing and urine was collected for 12 h overnight. Urine was tested by dipstick (Roche Diagnostics Corp., Indianapolis, IN, USA) for hematuria and leukocyturia and proteinuria and the extent is expressed as the mean on a scale of 0 (none) to 4 (severe). The albuminuria was determined by an albumin-specific enzyme-linked immunosorbent assay (CellTrend, Luckenwalde, Germany).

Histological evaluation of renal injury

Kidney tissue was collected at the time of killing and fixed in 10% formalin and embedded in paraffin using routine protocols. Coronal sections of specimens (4 μmol/l) were stained with hematoxylin and eosin, periodic acid Schiff's and evaluated by light microscopy. The extent of glomerular crescents and necrosis were expressed as the mean percent of glomeruli with crescents and necrosis in each animal. For immunofluorescence microscopy to detect glomerular localization of immune determinants, 4 μmol/l frozen sections were stained with fluoresceinated antibodies. The glomerular IgG deposition was performed by staining using fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Deposition of mouse complement C3, IgG, IgM, IgA, and MPO was visualized with a fluorescein isothiocyanate-conjugated goat anti-mouse C3, IgG, IgM, IgA, and MPO (ICN/Cappel, Aurora, OH, USA). Immunofluorescence microscopy staining of glomeruli was expressed as the mean intensity of staining for IgG, IgM, IgA, C3, and MPO on a scale of 0 to 4+. Glomerular neutrophil and macrophage influx was visualized using a neutrophil-specific mAb to GR-1 (BD Bioscience, Heidelberg, Germany) and a specific mAb to CD68 (AbD Serotec, Duesseldorf, Germany) as described earlier.[33]

Statistical analysis

Results are given as mean ± s.e.m. Comparisons between two groups were carried out using paired *t*-tests and between multiple groups with one- or two-way analysis of variance as indicated. Specific differences between multiple groups were then determined by use of a Bonferroni *post hoc* test. Differences were considered significant if $P<0.05$.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We thank Gisela Philipp, Baerbel Kuhlmann, and Sylvia Krueger for excellent technical assistance. This work was supported by a grant from the European Union, EuReGene, and the Deutsche Forschungsgemeinschaft (DFG: SCHR 771/5-1).

REFERENCES

1. van der Woude FJ, Rasmussen N, Lobatto S *et al.* Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1985; **1**: 425–429.
2. Franssen CF, Stegeman CA, Kallenberg CG *et al.* Antiproteinase 3- and antimyeloperoxidase-associated vasculitis. *Kidney Int* 2000; **57**: 2195–2206.
3. Falk RJ, Terrell RS, Charles LA *et al.* Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc Natl Acad Sci USA* 1990; **87**: 4115–4119.
4. Kocher M, Edberg JC, Fleit HB *et al.* Antineutrophil cytoplasmic antibodies preferentially engage Fc gammaRIIIb on human neutrophils. *J Immunol* 1998; **161**: 6909–6914.
5. Sibelius U, Hattar K, Schenkel A *et al.* Wegener's granulomatosis: anti-proteinase 3 antibodies are potent inductors of human endothelial cell signaling and leakage response. *J Exp Med* 1998; **187**: 497–503.
6. Ewert BH, Becker ME, Jennette JC *et al.* Antimyeloperoxidase antibodies induce neutrophil adherence to cultured human endothelial cells. *Ren Fail* 1995; **17**: 125–133.
7. Schreiber A, Luft FC, Kettritz R. Membrane proteinase 3 expression and ANCA-induced neutrophil activation. *Kidney Int* 2004; **65**: 2172–2183.
8. Xiao H, Heeringa P, Hu P *et al.* Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* 2002; **110**: 955–963.
9. Little MA, Smyth CL, Yadav R *et al.* Antineutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions *in vivo*. *Blood* 2005; **106**: 2050–2058.
10. Csernok E, Ernst M, Schmitt W *et al.* Activated neutrophils express proteinase 3 on their plasma membrane *in vitro* and *in vivo*. *Clin Exp Immunol* 1994; **95**: 244–250.
11. Kettritz R, Schreiber A, Luft FC *et al.* Role of mitogen-activated protein kinases in activation of human neutrophils by antineutrophil cytoplasmic antibodies. *J Am Soc Nephrol* 2001; **12**: 37–46.
12. Van Rossum AP, van der Geld YM, Limburg PC *et al.* Human anti-neutrophil cytoplasm autoantibodies to proteinase 3 (PR3-ANCA) bind to neutrophils. *Kidney Int* 2005; **68**: 537–541.
13. Ben-Smith A, Dove SK, Martin A *et al.* Antineutrophil cytoplasm autoantibodies from patients with systemic vasculitis activate neutrophils through distinct signaling cascades: comparison with conventional Fcgamma receptor ligation. *Blood* 2001; **98**: 1448–1455.
14. Choi M, Rolle S, Rane M *et al.* Extracellular signal-regulated kinase inhibition by statins inhibits neutrophil activation by ANCA. *Kidney Int* 2003; **63**: 96–106.
15. Kettritz R, Choi M, Butt W *et al.* Phosphatidylinositol 3-kinase controls antineutrophil cytoplasmic antibodies-induced respiratory burst in human neutrophils. *J Am Soc Nephrol* 2002; **13**: 1740–1749.
16. Williams JM, Pettitt TR, Powell W *et al.* Antineutrophil cytoplasm antibody-stimulated neutrophil adhesion depends on diacylglycerol kinase-catalyzed phosphatidic acid formation. *J Am Soc Nephrol* 2007; **18**: 1112–1120.
17. Hewins P, Williams JM, Wakelam MJ *et al.* Activation of Syk in neutrophils by antineutrophil cytoplasm antibodies occurs via Fcgamma receptors and CD18. *J Am Soc Nephrol* 2004; **15**: 796–808.
18. Radford DJ, Luu NT, Hewins P *et al.* Antineutrophil cytoplasmic antibodies stabilize adhesion and promote migration of flowing neutrophils on endothelial cells. *Arthritis Rheum* 2001; **44**: 2851–2861.
19. Harper L, Cockwell P, Adu D *et al.* Neutrophil priming and apoptosis in anti-neutrophil cytoplasmic autoantibody-associated vasculitis. *Kidney Int* 2001; **59**: 1729–1738.
20. Porges AJ, Redecha PB, Kimberly WT *et al.* Anti-neutrophil cytoplasmic antibodies engage and activate human neutrophils via Fc gamma RIIa. *J Immunol* 1994; **153**: 1271–1280.
21. Williams JM, Savage CO. Characterization of the regulation and functional consequences of p21ras activation in neutrophils by antineutrophil cytoplasm antibodies. *J Am Soc Nephrol* 2005; **16**: 90–96.
22. Koyasu S. The role of PI3K in immune cells. *Nat Immunol* 2003; **4**: 313–319.
23. Vanhaesebroeck B, Leevers SJ, Ahmadi K *et al.* Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* 2001; **70**: 535–602.
24. Hirsch E, Katanaev VL, Garlanda C *et al.* Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 2000; **287**: 1049–1053.
25. Li Z, Jiang H, Xie W *et al.* Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science* 2000; **287**: 1046–1049.
26. Sasaki T, Irie-Sasaki J, Jones RG *et al.* Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 2000; **287**: 1040–1046.
27. Sasaki T, Suzuki A, Sasaki J *et al.* Phosphoinositide 3-kinases in immunity: lessons from knockout mice. *J Biochem* 2002; **131**: 495–501.
28. Camps M, Ruckle T, Ji H *et al.* Blockade of PI3Kgamma suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat Med* 2005; **11**: 936–943.
29. Suire S, Condliffe AM, Ferguson GJ *et al.* Gbetagammas and the Ras binding domain of p110gamma are both important regulators of PI(3)Kgamma signalling in neutrophils. *Nat Cell Biol* 2006; **8**: 1303–1309.
30. Ferrandi C, Ardissone V, Ferro P *et al.* Phosphoinositide 3-kinase gamma inhibition plays a crucial role in early steps of inflammation by blocking neutrophil recruitment. *J Pharmacol Exp Ther* 2007; **322**: 923–930.
31. Barber DF, Bartolome A, Hernandez C *et al.* PI3Kgamma inhibition blocks glomerulonephritis and extends lifespan in a mouse model of systemic lupus. *Nat Med* 2005; **11**: 933–935.
32. Schreiber A, Xiao H, Jennette JC *et al.* C5a receptor mediates neutrophil activation and ANCA-induced glomerulonephritis. *J Am Soc Nephrol* 2009; **20**: 289–298.
33. Schreiber A, Xiao H, Falk RJ *et al.* Bone marrow-derived cells are sufficient and necessary targets to mediate glomerulonephritis and vasculitis induced by anti-myeloperoxidase antibodies. *J Am Soc Nephrol* 2006; **17**: 3355–3364.
34. al-Shami A, Bourgoin SG, Naccache PH. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. I.

Tyrosine phosphorylation-dependent stimulation of phosphatidylinositol 3-kinase and inhibition by phorbol esters. *Blood* 1997; **89**: 1035–1044.
35. Cadwallader KA, Condliffe AM, McGregor A *et al.* Regulation of phosphatidylinositol 3-kinase activity and phosphatidylinositol 3,4,5-trisphosphate accumulation by neutrophil priming agents. *J Immunol* 2002; **169**: 3336–3344.
36. Yasui K, Sekiguchi Y, Ichikawa M *et al.* Granulocyte macrophage-colony stimulating factor delays neutrophil apoptosis and primes its function through Ia-type phosphoinositide 3-kinase. *J Leukoc Biol* 2002; **72**: 1020–1026.
37. Little MA, Bhangal G, Smyth CL *et al.* Therapeutic effect of anti-TNF-alpha antibodies in an experimental model of anti-neutrophil cytoplasm antibody-associated systemic vasculitis. *J Am Soc Nephrol* 2006; **17**: 160–169.
38. Xiao H, Heeringa P, Liu Z *et al.* The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am J Pathol* 2005; **167**: 39–45.
39. Reumaux D, Vossebeld PJ, Roos D *et al.* Effect of tumor necrosis factor-induced integrin activation on Fc gamma receptor II-mediated signal transduction: relevance for activation of neutrophils by anti-proteinase 3 or anti-myeloperoxidase antibodies. *Blood* 1995; **86**: 3189–3195.
40. Calderwood JW, Williams JM, Morgan MD *et al.* ANCA induces beta2 integrin and CXC chemokine-dependent neutrophil-endothelial cell interactions that mimic those of highly cytokine-activated endothelium. *J Leukoc Biol* 2005; **77**: 33–43.
41. von Vietinghoff S, Eulenberg C, Wellner M *et al.* Neutrophil surface presentation of the anti-neutrophil cytoplasmic antibody-antigen proteinase 3 depends on N-terminal processing. *Clin Exp Immunol* 2008; **152**: 508–516.
42. Hannigan M, Zhan L, Li Z *et al.* Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during *N*-formyl-Met-Leu-Phe-induced chemotaxis. *Proc Natl Acad Sci USA* 2002; **99**: 3603–3608.
43. Konrad S, Ali SR, Wiege K *et al.* Phosphoinositide 3-kinases gamma and delta, linkers of coordinate C5a receptor-Fcgamma receptor activation and immune complex-induced inflammation. *J Biol Chem* 2008; **283**: 33296–33303.
44. Pick E, Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J Immunol Methods* 1981; **46**: 211–226.
45. Aratani Y, Koyama H, Nyui S *et al.* Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. *Infect Immun* 1999; **67**: 1828–1836.